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The Synthesis of N^4-(6-Aminopyridin-2-yl)-2′-deoxycytidine for Recognizing the CG Base Pair at Neutral pH by Oligodeoxyribonucleotide-Directed Triple Helix Formation

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The sequence-specific recognition of double-helical DNA by oligodeoxyribonucleotide-directed triple helix (triplex) formation is limited mostly to purine tracts. To interrupt the purine tract in a target sequence, a non-natural deoxyribonucleoside N^4-(6-aminopyridin-2-yl)-2′-deoxycytidine (^{P}C) was designed to interact with the C base in the CG base pair. The protected phosphoramidite synthon of ^{P}C was synthesized in seven steps and then was incorporated into an oligodeoxyribonucleotide by an automatic DNA synthesizer. Two 22-mers, designated as **C2** and **P**, with a common sequence of 5′-d-T^{m}CTXT^{m}CTTCTGTCTCCAGA-CAG were synthesized in this study. ^{m}C is 5-methyl-2′-deoxycytidine and X is either 2′-deoxycytidine (C) or ^{P}C for **C2** and **P**, respectively. **C2** is able to form a paper clip type triplex with one C · CG mismatched base triad in slightly acidic conditions but not at the neutral pH. On the other hand, **P** forms a stable triplex under both acidic and neutral conditions. This indicates that ^{P}C is able to form a ^{P}C · CG base triad in the triplex. Their physical properties were studied by UV thermal melting experiments and circular dichorism spectroscopy (CD). The thermal melting results imply that the ^{P}C · CG base triad is as stable as the C$^+$ · GC triad at pH 6.0, and ^{P}C helps the triplex formation preferably at neutral to acidic pH. In addition to the hydrogen bonding interaction with the CG base pair, the hydrophobic interaction of ^{P}C may also play an important role in stabilizing the triplex formation of oligodeoxyribonucleotides. In the presence of spermine at either pH 5.0 or pH 6.0, the melting temperature of the third strand of **P** was elevated about 30 and 21 °C, respectively. Thus, spermine can enhance the stability of the triple-helical structure.

INTRODUCTION

The studies of oligodeoxyribonucleotide-directed triple helix (triplex) formation have been widespread.[1,2] This is because the triplex formation can control gene expression or intervene in protein synthesis via so-called antigene and/or antisense oligodeoxyribonucleotides.[3] Currently, at least two classes of DNA triplexes, pyrimidine·purine·pyrimidine and purine·purine·pyrimidine motifs, have been studied extensively,[1,4] which differ in the sequence composition of the third strand and the relative orientations of the three strands. In these two triple-helical structures, the sequence-specific binding occurs by the formation of Hoogsteen/reverse Hoogsteen hydrogen bonds between bases in the third strand and duplex base pairs, predominantly at the purines of base pairs. Although purine sequences of considerable length are frequently found in human and eukaryotic genes,[5,6] these sequences are often interrupted by one or a few pyrimidine bases. Such (pyrimidine base) interruptions essentially reduce the triplex stability or even prevent triplex formation.[7,8] The range of sequences recognized by triplex-forming oligodeoxyribonucleotides could be expanded if means could be found to deal with these interruptions.

Various strategies have been developed to accommodate pyrimidine base interruptions. The linked oligodeoxyribonucleotides for alternate strand triplex formation were developed to extend the number of sites capable of being recognized.[9] The other approach was to simply skip the interruption, incorporating an abasic site into oligodeoxyribonucleotides at a site opposite a pyrimidine base interruption.[10] However, those resulted in considerable destabilization of the triplexes. Apparently, the continuous stacking interaction along the third strand is an important contributor to triplex stability.

Several nucleoside analogs have also been shown to recognize CG or TA base pairs, such as 2′-deoxynebularine,[11] N7-benzoyl derivative of 2′-deoxyformycin A,[12] and so on. 1-(2-Deoxy-β-D-ribofuranosyl)-4-(3-benzamidophenyl)imidazole could specifically recognize TA and CG interruptions.[13] This non-natural nucleoside was found

Dedicated to Professor Kung-Tsung Wang on the occasion of his 70th birthday.

to interact by sequence-specific intercalation, rather than by hydrogen bonding to the base pairs.

The pyrimidine base provides only one site for the hydrogen bond formation, thus destabilizing the Hoogsteen domain. Base analogs, which can potentially form hydrogen bonds with both bases of a pyrimidine·purine base pair, might enhance triplex stability and/or specificity at the site of an interruption. N^4-(6-Aminopyridin-2-yl)-2′-deoxycytidine (^{P}C) has an aminopyridinyl arm, sufficiently long to span the major groove of a duplex at a ^{P}C · CG binding site.[14] A binding mode is shown in Fig. 1, in which the imino tautomeric form of ^{P}C provides a hydrogen-bond acceptor site for the N4-H of C and the 6-amino group to interact with O6 of G. In acidic conditions, the protonated ^{P}C might give the possibility of forming an additional hydrogen bond between N1-H$^+$ of the aminopyridinyl group and O6 of G.

In this report, we describe the detailed syntheses of N^4-(6-aminopyridin-2-yl)-2′-deoxycytidine and the oligodeoxyribonucleotide containing this nucleoside. Under appropriate conditions, the ^{P}C-containing oligodeoxyribonucleotide can fold into a paper clip type triplex by itself. ^{P}C forms a stable base triad with a CG base pair. The physical properties were studied by measurement of CD and UV spectra.

RESULTS AND DISCUSSION

Syntheses of Protected ^{P}C and Oligodeoxyribonucleotides

The protected phosphoramidite of N^4-(6-aminopyridin-2-yl)-2′-deoxycytidine (^{P}C), suitable for incorporation into oligodeoxyribonucleotides by the automatic DNA synthesizer, was synthesized from 2′-deoxyuridine. A scheme for the synthesis of phosphoramidite **8** is depicted in Fig. 2. 2′-Deoxyuridine (**2**) was acetylated first with acetic anhydride in pyridine to give **3** as a white foam in a quantitative yield. The reaction mixture was chromatographed to remove the residual acetic acid that might interfere with the following reaction. Compound **3** was then reacted with phosphoryl chloride and 1,2,4-triazole in dry acetonitrile to yield 4-(1,2,4-triazol-1-yl) derivative **4** in 85% yield. The 1,2,4-triazol-1-yl group served as a good leaving group for the nucleophilic substitution reaction. **4** was reacted with the mono-protected N^2-benzoyl-2,6-diaminopyridine (**1**), which was prepared from 2,6-diaminopyridine separately, to give compound **5** with a moderate yield. The nucleophilic substitution by non-protected 2,6-diaminopyridine

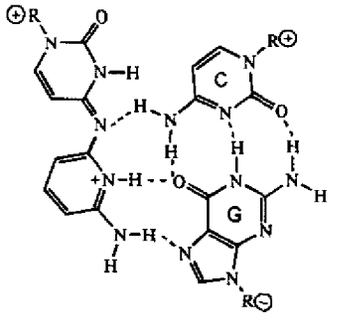

Fig. 1. A model for the ^{P}C · CG base triad.

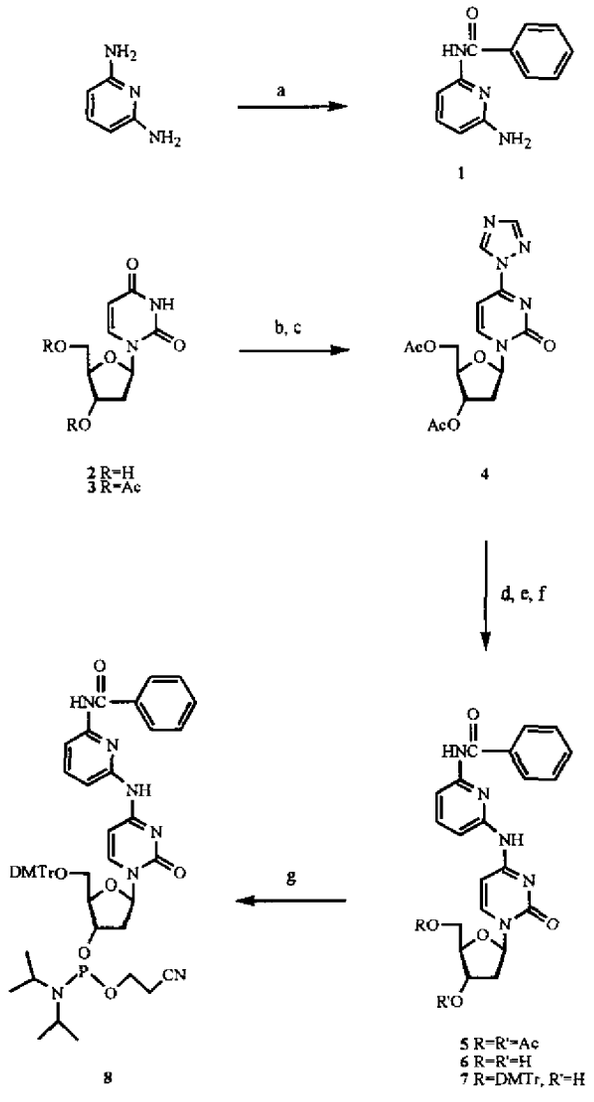

Fig. 2. Reagents and conditions: (a) BzCl, Et_3N, THF, -10 °C. (b) Ac_2O, pyridine, 0 °C. (c) 1,2,4-triazole, $POCl_3$, Et_3N, CH_3CN, 0 °C. (d) **1**, pyridine, reflux. (e) guanidine, EtOH/CH_2Cl_2 (9:1 v/v), r.t. (f) DMTrCl, pyridine, 35 °C. (g) 2-cyanoethyl *N,N*-diisopropylphosphoramidous chloride, diisopropylethylamine, CH_2Cl_2, r.t.

was also tried by the procedure from Huang et al.[14] However, we found that the target product was not only obtained in a low yield but also difficult to purify. The aromatic amino group of 2,6-diaminopyridine could be selectively benzoylated by controlling the amount of benzoyl chloride, reaction time and temperature to yield mono-protected benzamide **1**. The subsequent deprotection of the hydroxy groups in ethanolic guanidine gave **6**, which was recrystallized from methanol to afford a 81% yield in a white solid. Treatment of **6** with 4,4′-dimethoxytrityl chloride furnished the protected nucleoside **7**. Compound **7** was then activated with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidous chloride to afford phosphoramidite **8**, which was readily introduced into oligodeoxyribonucleotides.

All oligodeoxyribonucleotides were synthesized on a DNA synthesizer using standard phosphoramidite chemistry and purified as described previously.[15] The sequences of oligodeoxyribonucleotides are listed in Fig. 3. The coupling time during incorporation of the modified nucleoside ^{P}C into the oligodeoxyribonucleotide was extended to provide warranty for the yield. The deprotection of the oligodeoxyribonucleotide containing ^{P}C was treated with ammonium hydroxide/pyridine (1:1 v/v) and extended to 18 h to ensure the complete removal of the 6-benzamido protecting group from this nucleoside.

5′-d-T CT CT C TT CTCTCT CC AGAGAG-3′ C1
5′-d-T ^{m}CT CT ^{m}C TT CTGTCT CC AGACAG-3′ C2
5′-d-T ^{m}CT ^{P}CT ^{m}C TT CTGTCT CC AGACAG-3′ P
5′-d-T TT TT T TT CTGTCT CC AGACAG-3′ R

^{m}C = 5-methyl-2′-deoxycytidine
^{P}C = *N*⁴-(6-aminopyridin-2-yl)-2′-deoxycytidine

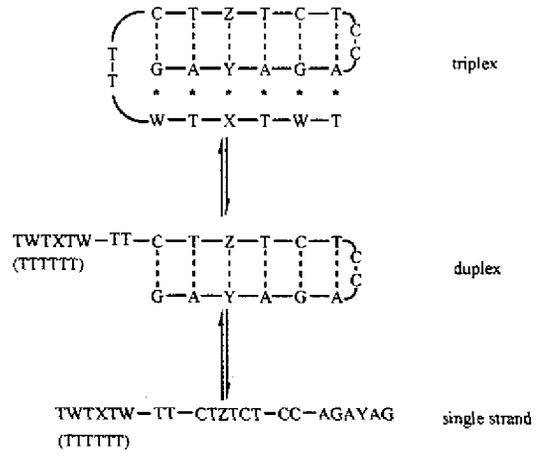

Fig. 3. A list of the synthetic oligodeoxyribonucleotides and the structure of a paper clip type triple helix.

Physical Study of Triple Helix Formation

Formation of a Paper Clip Type Triple Helix

The physical property of **C1** was studied previously by UV thermal melting experiments and CD spectra.[16] The results showed that a paper clip type triplex was formed in slightly acidic conditions (pH 4.5-5.0), but not at the neutral pH. CD spectra in a buffer of pH 5.0 with 150 mM sodium chloride and 20 mM magnesium chloride showed a characteristic band at 220 nm, but the band diminished when the pH was raised to 7.0 in the same buffer solution.[16] The changing of the pattern indicated dissociation of the Hoogsteen domain as expected. Thus, **C1** formed a triple helix in slightly acidic conditions, but gradually dissociated to a double helix upon raising the pH.

In a concentration-dependent study at pH 4.5, Tm values of **C1** were almost the same (60 ± 3 °C) in the entire concentration range from 0.4 to 400 μM.[16] This result indicated that the triplex formation was in an unimolecular way. In other words, **C1** formed a paper clip type triplex by self-folding.

C2 and **P** have similar sequences to **C1** as shown in Fig. 3. **C** · **GC** triads of **C1** are partially replaced by ^{m}**C** · **GC**, ^{P}**C** · **CG** or **C** · **CG**, where ^{m}**C** is 5-methyl-2′-deoxycytidine. The aforementioned established system was then applied to a study of the triplex formation of **C2** and **P**. It was observed that an oligodeoxyribonucleotide containing ^{m}**C** bound duplex DNA at the neutral pH with greater affinity than that with **C**.[17] However, N3 protonation of 5-methylcytosine base was still required for the formation of a stable base triad (the ^{m}**C**$^{+}$ · **GC** triad).[17] **C2** containing one **C** · **CG** mismatched triad was used as a comparison study. Oligomer **R** with a T_6 tail, parallel to the purine tract of the Watson-Crick duplex, was not able to form the stable base triad with the **GC** base pair. Thus, **R** was used as a reference and formed only the Watson-Crick duplex under any conditions studied here.

Triple Helix Formation in Neutral Conditions

The structural stabilities of these oligodeoxyribonucleotides can be revealed by UV thermal melting experiments. The results are summarized in Table 1. There are two sigmoid transitions which can be deduced from the UV absorbance versus temperature profile of **P**, as shown in Fig. 4, in the buffer solutions of neutral pH with the presence of magnesium chloride and/or spermine. The biphasic curve is

Table 1. The Tm of **C1**, **C2**, **P** and **R** in Buffer Solutions of pH 7.0 (A), pH 6.0 (B) and pH 5.0 (C). The Strand Concentration for All Above Oligodeoxyribonucleotides Is 5 μM

	sequence symbol	150 mM NaCl		150 mM NaCl 20 mM $MgCl_2$	150 mM NaCl 20 mM $MgCl_2$ 5 mM spermine
		Tm1	Tm2/Tm1	Tm2/Tm1	Tm2/Tm1
A	**C2**	57	66	66	66
	P	57	66	17/65	19/66
	R	57	64	67	67
B	**C2**	53	10/66	11/66	18/66
	P	53	13/65	14/66	35/67
	R	58	67	67	67
	C1			15/64	
C	**C2**	46	25/60	29/62	38/62
	P	47	24/60	27/62	57
	R	46	61	64	64

indicative of the triplex formation. The Hoogsteen domain is less stable than the Watson-Crick domain. Thus, the two transitions correspond first to dissociation of the third strand and second to dissociation of the Watson-Crick duplex. In consequence, two Tm values could be obtained (in Table 1). The higher Tm (Tm1) of **P** was almost the same as that of the corresponding **R** because Tm1 was derived from dissociation of a duplex to a single strand. The lower one (Tm2) was, thus, for the melting of the third strand. With the absence of magnesium chloride and spermine, **P** showed only one melting temperature (Tm1) of the value close to that of **R**. It implied that magnesium chloride and spermine did facilitate the triplex formation of **P**.

However, **C2**, with one C · CG mismatched triad, gave only a single transition in UV melting profiles at neutral pH with or without magnesium chloride and spermine (data not shown here). All Tm values of **C2** were close to that of **R** in the same conditions (in Table 1A). Thus, **C2** formed a double-helical structure only. It is important to point out that ^{P}C supports the stable triplex formation of oligodeoxyribonucleotides under appropriate conditions of the physiologically relevant pH, with recognition of the CG interruption of the purine tract.

The broad biphasic transition observed for **P** (in Fig. 4) suggested the presence of more than one distinct structure of the ^{P}C · CG base triad. The melting range of the first transition for dissociation of the third strand was from 10 to 50 °C, about twice the range of the second transition. It might be postulated that in addition to providing a rigid platform to position the hydrogen bonding groups, the planar aromatic rings of ^{P}C could also participate in the stacking interactions with neighboring bases. Alternatively, the aminopyridinyl ring of ^{P}C could intercalate into the duplex.

Triple Helix Formation in Acidic Conditions

The stability of the $^{m}C^{+}$ · GC base triad relies on the N3-protonation of the 5-methylcytosine base. The $^{m}C^{+}$ · GC triad is getting stable as pH goes down, thus helping stabilization of a triple-helical structure. Oligomer **C2** was a double-helical structure in a buffer solution of pH 7.0 with 150 mM sodium chloride, but formed a triplex at 10 and 25 °C as pH shifted to 6.0 and 5.0, respectively (in Table 1). This is just because, in acidic conditions, the protonated ^{m}C bases of **C2** formed stable triads with GC base pairs. The $^{m}C^{+}$ · GC triads contributed to the triplex stability of **C2** and circumvented the disturbance from a C · CG mismatched triad. Furthermore, it can been seen in Table 1C that all Tm2 values of **C2** and **P** at pH 5.0 were much higher than those at pH 6.0. This demonstrated that the increasing stability was closely related to the extent of protonation of ^{m}C. Consequently, the Hoogsteen domain associated better as the pH was lowered from 6.0 to 5.0. In contrast to the biphasic transition, a single transition was observed for dissociation of **P** in a buffer solution with spermine at pH 5.0 (57 °C for Tm1/Tm2 in Table 1C). It implied that **P** formed a stable triplex and dissociated to a single strand directly.[18]

In a buffer solution of pH 6.0 with 150 mM sodium chloride and 20 mM magnesium chloride, **P** and **C1** had almost the same Tm2 (in Table 1B). This revealed that the ^{P}C · CG triad was as stable as the C^{+} · GC triad at pH 6.0. Under the same condition, Tm2 values of **P** and **C2** were 14 and 11 °C, respectively. Here the $^{m}C^{+}$ · GC triads of **P** and **C2** helped the triplex formation. However, in the same buffer

solution of pH 7.0 (in Table 1A), **P** gave Tm2 of 17 °C while **C2** was not able to form a triplex. Obviously, oligodeoxyribonucleotides containing ^{P}C formed the triple-helical structure preferably at pH 7.0 to pH 6.0. The interactions of ^{P}C with the CG base pair and/or neighboring bases seemed slightly stronger in neutral conditions than in acidic conditions. Although an additional hydrogen bond is possibly provided at acidic pH, N1 protonation of the aminopyridinyl group of ^{P}C would not favor the triplex formation. It might be postulated that the hydrophobic force, involved in triplex-forming nucleic acids containing ^{P}C, might play an important role and also cause the broad melting transition of the third strand (in Fig. 4).

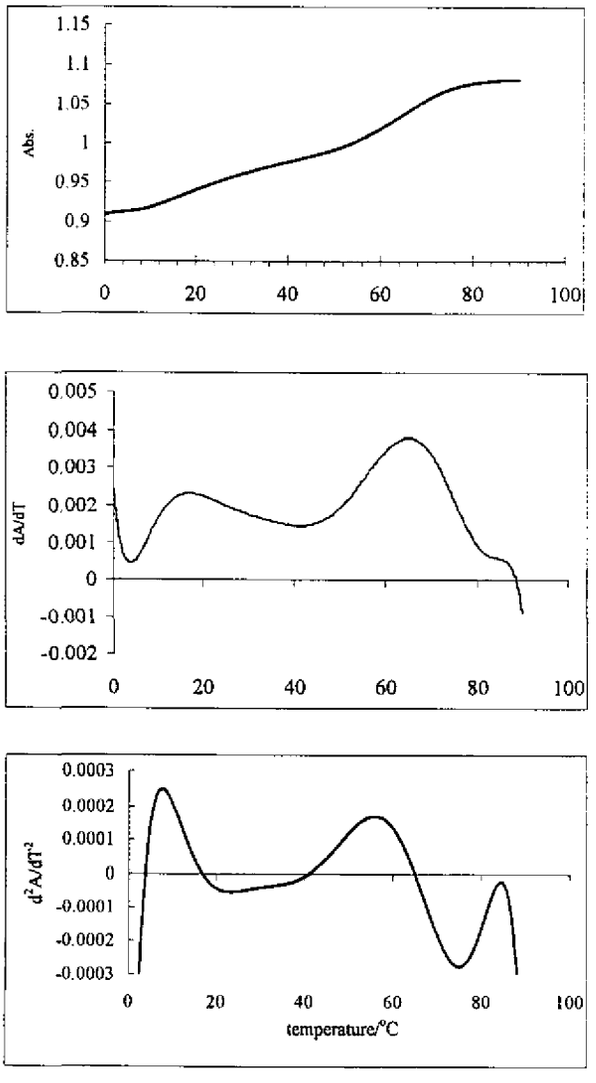

Fig. 4. The UV absorbance versus temperature profile (upper) of **P** in a buffer solution of pH 7.0 with 150 mM sodium chloride and 20 mM magnesium chloride. The strand concentration is 5 μM. The first-order differential (middle) and the second-order differential (lower) of the UV absorbance versus temperature plot.

It was observed in Table 1 that except for the absence of salts, all Tm1 values of the same pH fluctuated in a small range. This supported that Tm1 was the melting temperature of the Watson-Crick duplex because **C2**, **P** and **R** had the same duplex sequence. On the other hand, Tm2's of **C2** and **P** were elevated after adding magnesium chloride and/or spermine into the buffer solutions. Triplex **P** was 21 and 30 °C more stable in the presence of spermine at pH 6.0 and 5.0, respectively (Table 1B and 1C). Similar results were also obtained in **C2** with increasing about 7 to 9 °C at acidic pH. Thus, the magnesium cation and spermine could stabilize the triple-helical structure of oligodeoxyribonucleotides, and spermine, especially in acidic conditions, worked more effectively in enhancing the triplex formation to **P** than to **C2**.

Although oligodeoxyribonucleotide-directed triple helix formation offers a powerful chemical approach for the sequence-specific recognition of double-helical DNA, both pyrimidine and purine triple-helical motifs provide limited recognition codes and specify for mostly purine tracts of double-helical DNA. General methods for recognition of mixed sequences containing all four base pairs of duplex DNA are lacking. Our results showed that ^{P}C may be used to address the CG interruptions of the purine tracts in nucleic acid targets, thus extending the range of sequences recognized by triplex-forming oligodeoxyribonucleotides. This ability may prove useful in the design of novel antigene/antisense oligodeoxyribonucleotides.

EXPERIMENTAL SECTION

Materials

Chemicals were obtained from Aldrich or Sigma. Thin layer chromatography was performed on MN silica gel 60 F_{254} plates and column chromatography on MN silica gel G60. All reagents were used as obtained commercially.

The following buffers were used in CD and UV thermal melting experiments. Distilled, deionized water was used for all aqueous solutions. Buffer (pH range 5.0-6.0): 20 mM acetic acid, 20 mM sodium acetate; buffer (pH 7.0): 20 mM Tris-base, 20 mM Tris-acid.

General Physical Methods

Infrared (IR) spectra were recorded on a Perkin-Elmer 882 spectrophotometer. Standard spectra were regularly obtained for polystyrene to verify accuracy. ^{1}H NMR and ^{13}C NMR spectra were obtained using Bruker AC-200 and AC-

300 instruments. Chemical shifts were reported in ppm relative to the solvent residual signal or 85% phosphoric acid. Mass spectra were measured with a VG Analytical Model 70-250 s/se spectrometer. Melting point was recorded without correction on a melting point apparatus (MEL-TEMP II Laboratory Devices). The pH values of the samples were measured by a JENCO pH meter (Model 6071, Taiwan) with a microcombinational electrode (Ingold Electrode, Inc., USA). All oligodeoxyribonucleotides were synthesized on a DNA synthesizer (Applied Biosystems Model 391).

UV Melting

UV absorbance versus temperature profiles were measured by a JASCO V-560 UV/VIS spectrophotometer at 260 nm. The temperature was controlled by a JASCO TPU-436 temperature programmer and with a Peltier type thermostatic cell holder (EHC-441). Sample heating and cooling rates were 0.5 °C per minute. A cell with 10 mm path length were used. All oligodeoxyribonucleotides were prepared in a concentration of 5 μM.

CD Spectroscopy

CD spectra were obtained on a JASCO-720 circular dichroism spectropolarimeter. The temperature was controlled by a water-circulated jacketed cell. A cell with 10 mm path length were used. All oligodeoxyribonucleotides were prepared in a concentration of 5 μM.

N^2-benzoyl-2,6-diaminopyridine (1)

To a stirred solution of 2,6-diaminopyridine (0.5 g, 4.582 mmol) in THF (5 mL) was added triethylamine (0.7 mL). The mixture was cooled to -10 °C, followed by dropwise addition of benzoyl chloride (0.638 mL, 5.492 mmol). After 10 min, the reaction was quenched with methanol. The resulting solution was extracted with ethyl acetate and water. The organic layer was collected and dried over $MgSO_4$. The organic layer was evaporated and the residue was chromatographed on silica gel (chloroform/acetone, 15:1) to give a white solid 0.757 g (78%) of compound **1**. mp 146-147 °C; ^{1}H NMR ($CDCl_3$): δ 4.31 (br.s, 2H, NH_2), 6.27 (d, 1H, 5-H, $J_{4,5}$ = 7.9 Hz), 7.43-7.55 (m, 4H, 4-H, Ar-H), 7.70 (d, 1H, 3-H, $J_{3,4}$ = 7.9 Hz), 7.87 (d, 2H, Ar-H, J = 7.3 Hz), 8.23 (br.s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 103.4, 104.5, 127.1, 128.7, 132.0, 134.4, 140.1, 149.9, 157.1, 165.4; IR (KBr) (cm^{-1}): 3439, 3294, 3205, 1636, 1535, 1456, 1303, 1239, 788, 686; MS (rel. intensity): *m/z* 214 (MH^+, 100), 213 (M^+, 20), 196 (15), 185 (8), 136 (10), 105 (65), 93 (15), 77 (20); HRMS (EI) for $C_{12}H_{11}ON_3$ (M^+): calcd. 213.0902, found 213.0901.

3′,5′-*O*-diacetyl-2′-deoxyuridine (3)

2′-deoxyuridine (0.05 g, 0.219 mmol) in pyridine (0.565 mL) was cooled to 0 °C, and acetic anhydride (0.098 mL, 1.039 mmol) was added. The ice bath was removed and the progress of the reaction was followed by TLC (chloroform/acetone, 5:2). After 5 h, the solution was quenched with methanol, then evaporated under reduced pressure. The residue was chromatographed on silica gel (chloroform/acetone, 5:2) to give 0.0674 g (99%) of compound **3** as a white foam. ^{1}H NMR ($CDCl_3$): δ 2.07-2.17 (m, 7H, 2′-Ha, CH_3), 2.48 (dd, 1H, 2′-Hb, $J_{1',2'b}$ = 5.7 Hz, $J_{2'b,3'}$ = 1.9 Hz), 4.23-4.32 (m, 3H, 4′-H, 5′-Ha, 5′-Hb), 5.18 (m, 1H, 3′-H), 5.76 (d, 1H, 5-H, $J_{5,6}$ = 8.3 Hz), 6.26 (dd, 1H, 1′-H, $J_{1',2'a}$ = 8.3 Hz, $J_{1',2'b}$ = 5.7 Hz), 7.47 (d, 1H, 6-H, $J_{5,6}$ = 8.3 Hz), 9.58 (br.s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 20.7, 20.8, 37.8, 63.8, 74.0, 82.3, 85.3, 102.9, 138.8, 150.3, 163.1, 170.2, 170.3; IR (KBr) (cm^{-1}): 3509, 3216, 3058, 1734, 1697, 1463, 1380, 1240, 1109, 1057, 818, 766; MS (rel. intensity): *m/z* 313 (MH^+), 201 (15), 154 (15), 136 (20), 113 (52), 81 (100), 77 (10), 43 (37); HRMS (FAB) for $C_{13}H_{17}O_7N_2$ (MH^+): calcd. 313.1036, found 313.1041.

3′,5′-*O*-diacetyl-4-(1,2,4-triazol-1-yl)-2′-deoxyuridine (4)

A rapidly stirred suspension of 1,2,4-triazole (0.2 g, 2.88 mmol) in dry acetonitrile (1.56 mL) was treated at 0 °C by slow addition of phosphoryl chloride (0.056 mL, 0.616 mmol), followed by dropwise addition of triethylamine (0.362 mL). The suspension was left stirring for 30 min. To these was added a solution of compound **3** (0.1 g, 0.32 mmole) in dry acetonitrile (0.95 mL). The reaction mixture was stirred at room temperature overnight. Triethylamine (0.25 mL) and water (0.07 mL) were added. After 10 min, the solution was evaporated under reduced pressure and the residue was partitioned between chloroform and saturated aqueous sodium bicarbonate. The organic layer was collected, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was chromatographed on silica gel (chloroform/acetone, 3:1) to give 0.099 g (85%) of compound **4** as a white solid. mp. 151-152 °C; ^{1}H NMR ($CDCl_3$): δ 2.05-2.15 (m, 7H, 2′-Ha, CH_3), 2.90 (m, 1H, 2′-Hb, $J_{1',2'b}$ = 5.6 Hz, J_{gem} = 14.4 Hz, $J_{2'b,3'}$ = 2.2 Hz), 4.37-4.39 (m, 3H, 4′-H, 5′-Ha, 5′-Hb), 5.20 (m, 1H, 3′-H), 6.23 (dd, 1H, 1′-H, $J_{1',2'a}$ = 5.6 Hz, $J_{1',2'b}$ = 5.6 Hz), 7.07 (d, 1H, 5-H, $J_{5,6}$ = 7.2 Hz), 8.10 (s, 1H, triazole), 8.26 (d, 1H, 6-H, $J_{5,6}$ = 7.2 Hz), 9.23 (s, 1H, triazole); ^{13}C NMR ($CDCl_3$): δ 20.7, 20.8, 39.1, 63.5, 73.9, 83.4, 88.1, 94.6, 143.2, 145.4, 154.0, 159.4, 170.1, 170.3; IR (KBr) (cm^{-1}): 3117, 2960, 1742, 1663, 1553, 1514, 1467, 1425, 1377, 1241, 1115, 1057, 1028, 951, 891, 861, 783, 670; MS (rel. intensity): *m/z* 364 (MH^+), 307 (5), 289 (5), 201 (20), 164 (75), 81 (100), 77

(15), 43 (30); HRMS (FAB) for $C_{15}H_{18}O_6N_5$ (MH^+): calcd. 364.1257, found 364.1250.

3′,5′-*O*-diacetyl-N^4-(N^6-benzoyl-6-aminopyridin-2-yl)-2′-deoxycytidine (5)

A solution of compound **4** (1 g, 2.752 mmol) and compound **1** (1.172 g, 5.502 mmol) in pyridine (10 mL) was refluxed for one week, then evaporated under reduced pressure. The oil was extracted between chloroform and saturated aqueous sodium bicarbonate. The organic layer was collected, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was chromatographed on silica gel (chloroform/acetone, 5:1) to give 0.88 g (63%) of compound **5** as a yellow foam. ^{1}H NMR ($CDCl_3$): δ 2.04-2.14 (m, 7H, 2′-Ha, CH_3), 2.71 (m, 1H, 2′-Hb), 4.26-4.31 (m, 3H, 4′-H, 5′-Ha, 5′-Hb), 5.19 (m, 1H, 3′-H), 6.27 (dd, 1H, 1′-H, $J_{1',2'a}$ = 7.0 Hz, $J_{1',2'b}$ = 7.0 Hz), 6.48 (br.s, 1H, 5-H), 7.40-8.00 (m, 8 H, Ar-H), 8.67 (br.s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 20.8, 20.9, 38.7, 63.8, 74.3, 82.6, 86.9, 95.7, 109.6, 110.9, 127.3, 128.7, 132.3, 134.1, 140.7, 140.8, 149.9, 150.1, 155.1, 165.8, 170.3, 170.4; IR (KBr) (cm^{-1}): 3441 (br.), 3291 (br.), 3127, 2950, 1743, 1655, 1587, 1504, 1444, 1313, 1239, 1109, 1053, 795, 705; MS (rel. intensity): *m/z* 508 (MH^+), 308 (100), 307 (35); HRMS (FAB) for $C_{25}H_{26}O_7N_5$ (MH^+): calcd. 508.1832, found 508.1823.

N^4-(N^6-benzoyl-6-aminopyridin-2-yl)-2′-deoxycytidine (6)

Guanidine hydrochloride (0.02 g, 0.2 mmol) was added to a solution of sodium ethoxide (0.016 g, 0.24 mmol) in ethanol (0.2 mL). The mixture was stirred at room temperature for 30 min, then the solid was filtered. The filtrate was added to a solution of compound **5** in 1 mL ethanol/dichloromethane (9:1 v/v). After stirring at room temperature for 80 min, the solution was evaporated. The residue was chromatographed on silica gel (chloroform/methanol, 6:1), then recrystallized from methanol to afford a white solid 0.068 g (81%) of compound **6**. ^{1}H NMR (DMSO-d_6): δ 2.01 (m, 1H, 2′-Ha), 2.22 (ddd, 1H, 2′-Hb, $J_{1',2'b}$ = 6.5 Hz, J_{gem} = 13.1 Hz, $J_{2'b,3'}$ = 3.6 Hz), 3.56-3.60 (m, 2H, 5′-Ha, 5′-Hb), 3.83 (m, 1H, 4′-H), 4.23 (m, 1H, 3′-H), 5.01 (dd, 1H, 5′-OH, $J_{OH,5'a}$ = 5.1 Hz, $J_{OH,5'b}$ = 5.1 Hz), 5.22 (d, 1H, 3′-OH, $J_{OH,3'}$ = 4.3 Hz), 6.18 (dd, 1H, 1′-H, $J_{1',2'a}$ = 6.5 Hz, $J_{1',2'b}$ = 6.5 Hz), 7.07 (br.s, 1H, 5-H), 7.50-7.97 (m, 8H, Ar-H), 8.09 (d, 1H, 6-H, $J_{5,6}$ = 7.6 Hz), 10.16 (br.s, 1H, NH), 10.46 (br.s, 1H, NH); ^{13}C NMR (DMSO-d_6): δ 40.7, 61.2, 70.2, 85.5, 87.5, 90.4, 95.6, 109.1, 109.8, 127.9, 128.4, 131.8, 134.4, 139.8, 142.3, 150.1, 151.2, 154.5, 162.1, 166.0; IR (KBr) (cm^{-1}): 3390 (br.), 3294, 2930, 1644, 1587, 1444, 1310, 1086, 792, 702; MS (rel. intensity): *m/z* 424 (MH^+), 307 (100); HRMS (FAB) for $C_{21}H_{22}O_5N_5$ (MH^+): calcd. 424.1621, found 424.1620.

N^4-(N^6-benzoyl-6-aminopyridin-2-yl)-5′-dimethoxytrityl-2′-deoxycytidine (7)

To a stirred solution of compound **6** (0.1 g, 0.236 mmol) in pyridine (1 mL) were added 4,4′-dimethoxytrityl chloride (0.12 g, 0.355 mmol). The mixture was stirred for 6 h at 35 °C. The resulting solution was extracted with dichloromethane and saturated aqueous sodium bicarbonate. The combined organic layers were washed with brine, dried over $MgSO_4$, and evaporated. The residue was chromatographed (dichloromethane/acetone, 7:1) to furnish a yellow foam, then chromatographed again (dichloromethane/acetone/methanol, 40:15:1) to give 0.137 g (80%) of compound **7** as a white foam. ^{1}H NMR ($CDCl_3$): δ 2.23 (m, 1H, 2′-Ha), 2.64 (m, 1H, 2′-Hb), 3.34-3.48 (m, 2H, 5′-Ha, 5′-Hb), 3.70-3.79 (m, 7H, OCH_3, OH), 4.05 (m, 1H, 4′-H), 4.46 (m, 1H, 3′-H), 6.27-6.31 (m, 2H, 1′-H, 5-H), 6.76-6.79 (m, 4H, Ar-H), 7.12-8.06 (m, 18H, Ar-H), 8.44 (br.s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 41.9, 55.2, 62.7, 70.7, 86.0, 86.8, 95.2, 109.1, 110.5, 113.2, 127.0, 127.2, 127.9, 128.1, 128.7, 130.1, 132.2, 134.1, 135.4, 140.6, 142.3, 144.4, 149.8, 150.4, 158.5, 162.0, 165.7; IR (KBr) (cm^{-1}): 3416 (br.), 2930, 1649, 1582, 1506, 1445, 1308, 1249, 1175, 1092, 1033, 795, 705; MS (rel. intensity): *m/z* 726 (MH^+), 460 (15), 307 (100), 303 (75); HRMS (FAB) for $C_{42}H_{40}O_7N_5$ (MH^+): calcd. 726.2928, found 726.2914.

N^4-(N^6-benzoyl-6-aminopyridin-2-yl)-5′-dimethoxytrityl-2′-deoxycytidine 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylaminophosphoramidite) (8)

The compound **7** (0.05 g, 0.069 mmol) was dissolved in pyridine, and the solvent was concentrated in vacuo. This procedure was repeated twice. The residue was dissolved in CH_2Cl_2 (1.5 mL). Diisopropylethylamine (0.06 mL, 0.345 mmol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidous chloride (0.046 mL, 0.207 mmol) were added to the solution. The whole was kept at room temperature for 1 h, then partitioned between $CHCl_3$ and 5% aqueous sodium bicarbonate. The organic layer was separated and dried over Na_2SO_4. The residue was chromatographed on silica gel (dichloromethane/ethyl acetate, 2:1, with 1% triethylamine) to give 0.047 g (73%) of the phosphoramidite **8** as a white foam. ^{1}H NMR ($CDCl_3$): δ 1.07-1.26 (m, 12H, *i*-Pr), 2.27 (m, 1H, 2′-Ha), 2.52-2.74 (m, 3H, 2′-Hb, CH_2CN), 3.35-3.56 (m, 4H, 5′-Ha, 5′-Hb, NCH), 3.71-4.62 (m, 10H, 3′-H, 4′-H, OCH_2, OCH_3), 6.11 (d, 1H, 5-H, $J_{5,6}$ = 6.3 Hz), 6.30 (dd, 1H, 1′-H, $J_{1',2'a}$ = 5.8 Hz, $J_{1',2'b}$ = 5.8 Hz), 6.76-6.82 (m, 4H, Ar-H), 7.15-8.06 (m, 18H, Ar-H), 8.45 (br.s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 20.0, 20.1, 20.3, 20.4, 22.9, 24.5, 43.1,

43.3, 45.3, 45.4, 55.2, 57.8, 58.1, 58.2, 58.3, 62.4, 63.0, 72.3, 72.5, 84.8, 85.2, 85.3, 86.5, 86.8, 86.9, 95.0, 109.1, 109.5, 110.6, 113.2, 116.8, 117.5, 118.3, 127.0, 127.2, 127.5, 127.9, 128.2, 128.7, 130.1, 132.0, 132.2, 134.2, 134.6, 135.2, 135.4, 135.6, 140.4, 140.8, 142.2, 144.3, 149.6, 149.8, 150.3, 155.2, 158.6, 161.8, 165.6; ^{31}P NMR ($CDCl_3$): δ 147.5; IR (KBr) (cm^{-1}): 3414, 3074, 2985, 2235, 1655, 1596, 1507, 1448, 1318, 1181, 1124, 1081, 980; MS (rel. intensity): *m/z* 926 (MH^+), 708 (5), 303 (100); HRMS (FAB) for $C_{51}H_{57}O_8N_7P$ (MH^+): calcd. 926.4006, found 926.4009.

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N^4-(6-Aminopyridin-2-yl)-2′-deoxycytidine; ^{P}C · CG Base triad; A paper clip type triple helix; Oligodeoxyribonucleotide.

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